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RESEARCH PAPER

Activation of apoptotic cell death by skin mucus from Asian swamp eel (*Monopterus albus*) against human lung cancer cell line

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تنشيط موت الخلايا المبرمج بالمخاط الجلدي من ثعبان الأنقليس الآسيوي (*Monopterus albus*) ضد خط خلايا سرطان الرئة البشرية

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Abstract. Asian swamp eel (*Monopterus albus*) is a freshwater fish which distributed widely in the East of India mainly across Malay Peninsula and Indochinese Peninsula, it is also broadly distributed in the Southern areas of East Asia including, southeastern China, Western Japanese Archipelago, and Korean Peninsula. It lives in muddy places, rice paddies, and slow-flowing currents areas. It has a unique morphological elongated body which is similar to snake and covered with a thick layer of mucus. The objective of this study is to screen the cytotoxic activity of eel skin mucus extracts and to evaluate the mechanism of cell death apoptosis or necrosis based on caspases activity assays. For this purpose eel skin mucus was collected from healthy fresh eels to prepare aqueous and methanol extracts. Preliminary cytotoxicity study was demonstrated against Non-small-cell lung carcinoma cell line (A549) using cell viability assay which revealed that methanol extract was more potent than aqueous extract based on the response of ESM methanol and aqueous extracts to the relevant IC_{50} concentrations as IC_{50} values were 621±0.09 µg/mL and 845 ± 0.25 µg/mL respectively. Subsequently cell death was induced through triggering apoptosis by the activation of caspase-3/7, 8 and 9. In conclusion, the present study has revealed the cytotoxic potentials of eel skin mucus which may lead to the development of new anticancer agents.

Keywords: *Monopterus albus*; cytotoxic activity; apoptosis; caspases.

المستخلص: أنقليس المستنقعات الآسيوية (*Monopterus albus*) هو من أسماك المياه العذبة التي يتم توزيعها على نطاق واسع في شرق الهند بشكل رئيسي عبر شبه جزيرة الملايو وشبه جزيرة الهند الصينية ، كما أنها موزعة بشكل واسع في المناطق الجنوبية من شرق آسيا بما في ذلك ، جنوب شرق الصين ، أرخبيل غرب اليابان ، و شبه الجزيرة الكورية. تعيش في الأماكن الموحلة وحقول الأرز ومناطق التيارات بطيئة التدفق. ولها تركيبة جسم فريدة من نوعها والتي تشبه فيها الثعبان وتغطى بطبقة سميكة من المخاط. الهدف من هذه الدراسة هو فحص النشاط السام على الخلايا لمستخلصات مخاط جلد ثعبان الأنقليس ولتقييم آلية موت الخلية سواء كان موت الخلايا المبرمج أو النخر على أساس اختبارات نشاط الكاسبيزس. لهذا الغرض؛ تم جمع مخاط جلد ثعبان البحر من ثعابين طازجة صحية لإعداد مستخلصات الماء والميثانول. تم إجراء دراسة أولية للسمية الخلوية ضد الخلايا غير الرئوية لسرطان الرئة (A٥٤٩) باستخدام اختبار قابلية الخلية التي أظهرت أن مستخلص الميثانول أقوى من المستخلص المائي حيث أن قيم IC_{50} كانت ٦٢١ ± ٠,٠٩ ميكروجرام / مل و ٨٤٥ ± ٠,٢٥ ميكروجرام / مل على التوالي. وفي وقت لاحق ، فإن آلية موت الخلية تسبب الموت الخلوي من خلال التسبب في موت الخلايا المبرمج عن طريق تفعيل الكاسبيز ٣/٧ و ٨ و ٩. في الختام ، كشفت الدراسة الحالية عن إمكانات الخلايا السامة للخلايا لمخاط ثعبان البحر مما قد يؤدي إلى تطور جديد. وكلاء مضاد للسرطان.

الكلمات المفتاحية: *Monopterus albus* ، النشاط السام للخلايا ، موت الخلايا المبرمج ، الكاسبيزس.

Introduction

Asian swamp eel (*Monopterus albus*) belongs to the family of Synbranchidae under Synbranchiformes order (Cheng et al., 2003). Asian swamp eel skin mucus is secreted by the epidermal goblet cells in the epidermis which composed from inorganic salts, immunoglobulins, lipids and gel-forming macromolecules such as mucins, and other glycoproteins suspended in water (Bragadeeswaran and Thangaraj, 2011), which gives the mucus lubricating properties (Pearson and Brownlee, 2005).

There are two main mechanisms describing the cell death in eukaryotic cells, apoptosis and necrosis. Apoptosis is a process with well-defined key steps that mark the progress of the process in individual cells. Cells undergoing apoptosis possess distinctive morphological, biochemical and molecular features including sequence of chromatin margination and aggregation, nuclear and cytoplasmic condensation, cellular shrinkage, budding and fragmentation through the partition of cytoplasm and nucleus into the apoptotic body (Eriksson et al., 2008). these apoptotic bodies immediately recognized and phagocytized by macrophages or adjacent epithelial cells. Hence, there is no inflammatory response is elicit-

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Table 1. Caspase-3/7 activity after treatment of A549 cells with ESM methanol and aqueous extracts along with positive control (Taxol) and negative control (untreated cells) for 24 hrs.

Treatment	OD1	OD2	OD3	Average	SD	Fold Change
Methanol	0.301	0.339	0.322	0.320*	0.019	3.340*
Aqueous	0.236	0.202	0.229	0.222*	0.017	2.344*
Taxol	0.351	0.332	0.347	0.343*	0.010	3.597*
Control	0.091	0.098	0.093	0.094	0.003	0.094

*Caspase-3/7 activities were determined using the CaspAce® system. Mean ± SD (n = 3 wells/treatment). *p < 0.05 compared with the untreated cells. Fold changes was calculated based on the control/untreated cells. OD is optical density.*

ed (Fadok et al., 2000). However, *in vitro*, the apoptotic bodies and residual cell fragments swell and lyse (Chang and Yang, 2000). The morphologic features that characterize apoptotic cells are the consequence of several biochemical features, which are stimulated by proteolytic destruction of cytoskeletal and metabolic proteins. Activation of the effector caspases 3 and 7 is a common step in both intrinsic and extrinsic apoptotic signal pathways, which accomplish the characteristic changes in the nuclear morphology and biochemistry, including chromatin condensation and DNA fragmentation (Fan et al., 2005).

Methodology

Materials

Cell Lines

The cell lines used in the study include human non-small lung carcinoma (A549, ATCC CCL-185) and normal mouse embryonic fibroblast (3T3-L1, ATCC CRL-3242). The cell lines were obtained from Biomedical Science Department, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia.

Chemicals

Phosphate buffered saline (PBS), Fetal bovine serum (FBS), Dulbecco`s modified Eagle medium (DMEM), Penicillin-streptomycin were purchased from Gibco Invitrogen Co. (Scotland, UK). Paclitaxel (Taxol®) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Merck KGaA, Germany. Caspase-3/7, caspase-8, caspase-9 kits were purchased from Promega, Madison, WI, USA.

Methods

Sample Collection and Extraction

The eel skin mucus (ESM) was collected from healthy eels by gently scraping the surface of the eel skin and then it was homogenized with 2 volumes of distilled water using homogenizer, followed by centrifugation at 13,000 rpm for 30 min, the supernatant was freeze-dried for 5 days. The dried substance was weighed and dissolved in distilled water to form aqueous extract and in methanol to form methanol extract, after that, the dissolved substance was filtered using 0.22 µm syringe filter to be ready for use. The extraction procedure was carried out according to the method previously described by Sadakane et al. (2007) with a slight modification.

Cell viability test (MTT-based cytotoxicity assay)

The antiproliferative effect of aqueous and methanol extracts of ESM on growth of two human cancer cell lines, i.e. human non-small lung carcinoma (A549) and normal mouse embryonic fibroblast (3T3-L1), were evaluated by MTT assay. Approximately 5×10^4 of cells were seeded into 96-well plates. after the cells reach the confluency level, they were treated with different concentrations of ESM aqueous and methanol extracts from 200 to 1000 µg/mL for 24 hrs. Then, 20 µl of MTT was added to each well and the plates were further incubated for 24 hrs. After that, 100 µl of DMSO was added to each well

Table 2. Caspase-8 activity after treatment of A549 cells with ESM methanol and aqueous extracts along with positive control (Taxol) and negative control (untreated cells) for 24 hrs.

Treatment	OD1	OD2	OD3	Average	SD	Fold Change
Methanol	0.380	0.371	0.295	0.348*	0.046	3.233*
Aqueous	0.197	0.229	0.215	0.213*	0.016	2.216*
Taxol	0.153	0.202	0.197	0.184*	0.026	1.790*
Control	0.103	0.109	0.115	0.109	0.006	0.109

*caspase-8 activities were determined using the CaspAce® system. Mean ± SD (n = 3 wells/treatment). *p < 0.05 compared with the untreated cells. Fold changes was calculated based on the control/untreated cells. OD is optical density.*

Table 3. Caspase-9 activity after treatment of A549 cells with ESM methanol and aqueous extracts along with positive control (Taxol) and negative control (untreated cells) for 24 hrs.

Treatment	OD1	OD2	OD3	Average	SD	Fold Change
Methanol	0.442	0.450	0.447	0.446*	0.004	3.937*
Aqueous	0.299	0.318	0.271	0.272*	0.023	2.725*
Taxol	0.541	0.519	0.528	0.529*	0.011	4.625*
Control	0.019	0.012	0.017	0.033	0.003	0.033

*caspase-9 activities were determined using the CaspAce® system. Mean ± SD (n = 3 wells/treatment). *p < 0.05 compared with the untreated cells. Fold changes was calculated based on the control/untreated cells. OD is optical density.*

and incubated for 4 hrs. Then, the absorbance was measured at 570 nm using a microplate reader. The percentage of cell survival rate was calculated using to the cell viability formula: Percentage of cell viability = (Mean of experimental absorbance / Mean of control absorbance) × 100 (Villarroel et al., 2007).

PBS was used as a negative control while 1 µg/mL of Taxol was used as a positive control as it is a chemotherapy medication used in the treatment of non-small-cell lung cancer (NSCLC) such as A549 cell line.

Determination of the mechanism of cell death by measuring of caspase 3, 8 and 9 activities

Caspase 3, 8 and 9 are members of the cysteine aspartic acid-specific protease (caspase) family which play key roles in apoptosis induction. The test was conducted according to the instructions of the kits. A549 cells were cultured in 96-well plates at the density of 1×10^4 cells per well overnight. The adherent cells were further incubated for 24 hrs with 100 µl media containing IC50 values of ESM aqueous and methanol extracts. After the treatment, the cells were harvested and centrifuged then the pellets were washed with PBS and lysis in chilled lysis buffer. The mixture was left on ice for 10 min then centrifuged at 2000 rpm for 5 min at 4°C. Then the supernatant was used for the determination of caspase activities. The results were read on a microplate reader at 405 nm (Abdullah et al., 2015).

Results

Cell viability assay

The results showed that the reduction in cell viability of A549-treated with ESM extracts was significant compared with the untreated cells. At 200 µg/mL which was the lowest concentration used, the cell viability of ESM aqueous and methanol was recorded as 87. 61% and 76.29% respectively whereas at 1000 µg/mL of ESM aqueous extract the cell viability was recorded as 46.58% while for methanol extract was 38.19%. The cell viability of ESM aqueous and methanol extracts against 3T3-L1 at 1000 µg/mL was 79.24% and 63.65% respectively while at 200 µg/mL was 92.53% and 89.21% respectively. The inhibitory concentrations of ESM extracts against A549 which is required to reduce 50% of cell viability (IC_{50}) was calculated and it was recorded as follows; 621 ± 0.09 µg/mL for methanol extract, 845 ± 0.25 µg/mL for aqueous extract and 43.12 ± 0.6 µg/mL for Taxol.

Effects of ESM aqueous and methanol extracts on caspase 3,8 and 9 activities

A549 cells-treated with the ESM extracts exhibited a sharp increase in the activities of caspases 3, 8 and 9. The activities of the individual caspases were expressed as fold increases with respect to the untreated control. Fold change is defined as the ratio between different values, the fold change of caspase 3,8 and 9 was higher for ESM methanol extract than aqueous extract compared to the control (untreated A549 cells) as shown in table 3.1,2,3. The activities of 3, 8 and 9 caspases were increased significantly in A549 cells-treated with 600 µg/mL of ESM methanol extract and 800 µg/mL of ESM aqueous extract compared to untreated cells (the concentration of methanol and aqueous extracts was chosen based on the nearest concentration to IC_{50} value which was 621 ± 0.09 µg/mL for ESM methanol extract and 845 ± 0.25 µg/mL for ESM aqueous extract.

Discussion

Treatment of ESM aqueous and methanol extracts significantly inhibited the growth of A549 cell line compared to the normal cell line. This result agrees with what has been reported about the anticancer activities of Amphibian skin mucus (Kawasaki and Iwamuro, 2008). The present results showed that ESM extracts treatments activated 3, 8 and 9 caspases compared to the control (untreated cells), indicating that they induced A549 cell death via apoptosis as caspases test one of the biochemical markers which can be used to distinguish the mechanism of cell death, as it activated in the case of both intrinsic and extrinsic apoptotic signals (Fan et al., 2005). However, necrotic cell death does not involve caspase activation (Mansilla et al., 2006). Activation of caspases consider a hallmark of apoptosis and there are two pathways included; death receptor pathway which based on caspase-8 activation and mitochondrial pathway which based on caspase-9 activation (Leong et al., 2016). Accordingly, activities of the caspases 3, 8 and 9

Table 4. Caspase-8 activity after treatment of A549 cells with ESM methanol and aqueous extracts along with positive control (Taxol) and negative control (untreated cells) for 24 hrs.

Treatment	OD1	OD2	OD3	Average	SD	Fold Change
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Taxol	0.153	0.202	0.197	0.184*	0.026	1.790*
Control	0.103	0.109	0.115	0.109	0.006	0.109

*caspase-8 activities were determined using the CaspAce® system. Mean ± SD (n = 3 wells/treatment). *p < 0.05 compared with the untreated cells. Fold changes was calculated based on the control/untreated cells. OD is optical density.*

were measured in A549 cells-treated with ESM aqueous and methanol extracts as well as Taxol which is a chemotherapy drug use for apoptosis induction (Hu et al., 2005).

Conclusion

The activation of caspase (3, 8 and 9) only occurs as a result of apoptosis not necrosis and this parameter has been considered as one of the most reliable biochemical parameters to differentiate between apoptotic and necrotic cell death, therefore, the current study revealed that ESM extracts activate cell death via apoptosis.

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